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Dated 3 October 2003

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1/77
10SEP02 E746918-8 D02890
P01/7700 0.00-0220894.0

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
South Wales
NP9 1RH

1. Your reference

REP07248GB

2. Patent application number
(The Patent Office will fill in this part)

0220894.0

- 9 SEP 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Millipore UK Ltd.
Unit 31
Medomsley Road Industrial Estate
Consett
Co. Durham
DH8 6SZ

Patents ADP number *(if you know it)* 8466883001

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

METHOD OF SEPARATION

5. Name of your agent *(if you have one)*

Gill Jennings & Every

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
London
EC2M 7LH

Patents ADP number *(if you know it)* 745002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

Patents Form 1/77

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Continuation sheets of this form	
Description	5
Claim(s)	1
Abstract	
Drawing(s)	2 + 2

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	NO

11. For the applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature

Date
9 September 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

PERRY, Robert Edward
020 7377 1377

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Notes

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b) Write your answers in capital letters using black ink or you may type them.

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d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

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METHOD OF SEPARATION

Field of the Invention

The present invention relates to a method of separation.

Background of the Invention

Under certain conditions, adsorbent materials containing ionic groups can bind molecules of a net opposing charge. Such processes, for example chromatography, are currently utilised in the purification and separation of biomolecules in a complex mixture such as blood or fermentation or cell culture broths.

In column chromatography, the mixture to be analysed is applied to the top of a column comprising an adsorbent material, the "stationary phase". A liquid solvent (the "mobile phase") is passed through the column by gravity or pressure carrying the dissolved mixture. Because the different compounds in the mixture have different ionic interactions with the mobile and stationary phases, they will be carried along in the mobile phase to varying degrees resulting in separation. A salt gradient is then usually applied to remove, in turn, separate bound components.

In such processes, the exact conditions for separation are typically determined by trial and error. The operational selectivity of an adsorbent relates to the number of molecules that bind to it as the mixture passes over; under normal conditions there is generally low specificity. Variation in the pH or ionic strength causes the interaction between individual components of the mixture and adsorbent to change. The ionic strength may be varied to allow a desired component to adsorb, but so that solvent molecules or additional components compete for available binding sites on the adsorbent, thus preventing the binding of an undesired component.

Selectivity also changes with the physical structure of the adsorbent, for example the size distribution of pores or the chemical nature of the underivatised adsorbent. Examples of such underivatised adsorbents include ceramic, synthetic or natural polymers.

Current methodologies suffer from a number of limitations, one of which is cost. A major factor in the manufacture of biomolecules is the cost of raw

materials. Buffered solutions are required to stabilise biomolecules against variations in pH and to reduce the likelihood of insolubilisation (precipitation). For this reason, concentrations of buffer/salt components are kept to a minimum in feed solutions and usually range from 10 to 100 mM, depending on the biomolecule to be stabilised.

Summary of the Invention

The present invention is based on the realisation that the selectivity of an adsorbent containing ionic groups can be optimised by varying the ionic charge density of the binding surfaces of the adsorbent.

According to an aspect of the invention, a method of separating a selected ionic component from a sample, comprises contacting the sample with an ionic adsorbent whose charge density is such that the component is bound selectively, in the absence of an added ionic component that competitively binds the adsorbent. When the adsorbent material is cationic, the material preferably comprises sulphopropyl groups which may be supported on a substrate, for example agarose or Sepharose.

A method of the invention may be used in the production of a monoclonal or polyclonal antibody, since antibody production generally requires a protein-specific purification step, such as protein A purification.

By optimising the charge density of the adsorbent surface, the invention provides a highly selective method of separation. A method of the invention involves a marked reduction in the amount of adsorbent-bound containing molecules, allowing greater working capacities to be attained within typical ranges of pH and ionic strength. In addition, cost is reduced, since the use of large quantities of raw materials, such as salts or buffer solutions, is unnecessary.

Description of Preferred Embodiments

The invention bypasses the need for the inclusion of competing ionic components (e.g. salts) in the sample. Selectivity is achieved by using an adsorbent of predetermined charge density suitable for selective binding of the ionic component of interest over a range of ionic strengths. Thus a sample may be brought directly into contact with the adsorbent, without any pretreatment.

Selectivity may be achieved by using an adsorbent of low enough ionic charge density such that only the component of interest binds to it. An example of this is the separation of a mixture of immunoglobulin (IgG) and protein A. Sulphopropyl (SP) groups having an (cat)ionic strength of >140 µmol/ml bind both proteins and the IgG-protein A complex. Sulphopropyl groups having weaker charge density, of approximately 75 µmol/ml, are selective towards IgG, the result being that, after elution, protein A is observed in the unbound fraction and IgG in the bound fraction.

The apparatus used for separation may be any suitable apparatus known in the art, for example an ion-exchange column. Techniques such as polyacrylamide gel electrophoresis (PAGE), in particular sodium dodecyl sulphate (SDS)-PAGE, may be used to analyse the various fractions of separation.

The present invention may be used for the separation of ionic polymeric compounds. In particular, the present invention may be used for the separation of biomolecules such as those found in complex mixtures such as blood and cell culture broths.

The following Examples illustrate the invention.

Example 1: separation of immunoglobulin and protein A

Agarose beads were manufactured, cross-linked and chemically derivatised with sulphopropyl (SP) groups of varying charge densities. A commercially available control SP adsorbent, SP Sepharose, was also used.

Immunoglobulin G and protein A were mixed in the ratio 10:1 (w/w) in a buffered solution, pH 4.0-5.5 and conductivity 2-6 mSi/cm. Three different SP-based cation-exchange adsorbents were analysed for selectivity of binding. These contained 75 µmol/ml cationic groups, 140 µmol/ml and SP Sepharose containing 200-250 µmol/ml cationic groups.

One column volume of the protein mixture was applied to a packed column of buffer-equilibrated SP adsorbent at a flow rate of 100 to 300 cm/hr. The column was then washed with buffered solution to remove non-bound protein, and a salt gradient of increasing conductivity was applied to the column in order to elute ionically-bound proteins.

The eluent from the column was analysed for absorbance at 220 nm using an on-line detector. A variety of pH and conductivity values were utilised within the ranges defined above. Non-bound fractions and bound-eluted fractions were retained from each column run, and analysed for protein content using SDS-PAGE.

The results are shown in Figure 1. The SP adsorbents with >140 µmol/ml cationic groups bound all the protein components; IgG, protein A and the complex between IgG and protein. However, the adsorbent with only 75 µmol/ml (dashed line) shows a substantial quantity of protein in the unbound fraction; this non-binding protein fraction was shown to be approximately 90% protein A and 10% IgG. The eluting protein fraction was shown to contain 98% IgG and 2% protein A. It is evident that the cationic adsorbent with lower charge density has not bound protein A.

Example 2: separation of acidic cheese whey proteins

Cheese whey was obtained from de-fatted milk and the pH adjusted to 4.3 with dilute phosphoric acid. One column volume of the protein mixture was applied to three SP cationic adsorbents in order to separate the different proteins. The proteins were washed from the column with a buffer solution of pH 4.3. The proteins were eluted from each adsorbent column using a gradient of increasing sodium chloride concentration (0 to 1M). The salt gradient was applied along each adsorbent column at an elution volume range of 12 to 50 ml. The eluent from the column was analysed for absorbance at 280 nm using an on-line detector.

Figure 2 shows three elution profiles (optical density at 280 nm versus elution volume) conducted using the three different cation adsorbents under the same binding and elution gradient conditions. The three cationic adsorbents had different ionic charge densities. The highest charge density adsorbent was Sepharose SP, followed by cross-linked agarose with 140 µmol/ml cationic SP groups, followed by cross-linked agarose with 75 µmol/ml cationic SP groups. The optical density traces show how the acidic whey proteins were bound and eluted from each adsorbent.

The absorbance profiles show two proteins separated into distinct peaks for the adsorbents having >100 µmol/ml ionic charge density. The adsorbent with < 100 µmol/ml ionic charge density bound and eluted only one protein. This latter adsorbent has changed its binding specificity and become more selective in operation.

CLAIMS

1. A method of separating a selected ionic component from a sample, which comprises contacting the sample with an ionic adsorbent whose charge density is such that the component is bound selectively in the absence of added ionic component that competitively binds the adsorbent.

2. A method according to claim 1, wherein the adsorbent is cationic.

3. A method according to claim 2, wherein the adsorbent comprises a sulphopropyl group.

4. A method according to any preceding claim, wherein the charge density of the adsorbent is 50 to 100 µmol/ml.

5. A method according to any preceding claim, wherein the selected ionic component is a protein.

6. A method according to claim 5, wherein the protein is an immunoglobulin.

7. A method according to any preceding claim, wherein the sample comprises protein A.

Figure 1 – Separation of Immunoglobulin and protein A protein species.

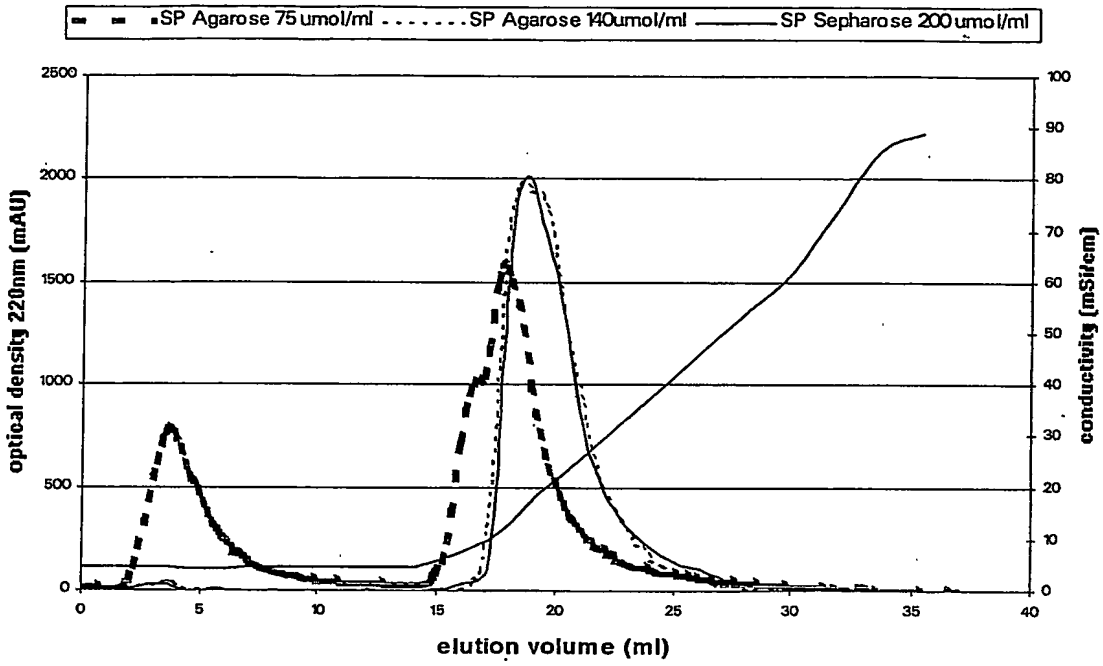

Figure 2. Resolution of acidic cheese whey proteins.